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Original Article

Clinical significance of exosomal long noncoding RNA DANCR as a novel serum-based diagnostic and prognostic biomarker in osteosarcoma

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Abstract: Purpose: The present study aimed to explore the clinical significance of lncRNA DANCR in osteosarcoma patients. Methods: This study included four parts: 1) Expression profiles of exosomal DANCR in osteosarcoma patients and cells; 2) The stability of exosomal DANCR; 3) The origin of exosomal DANCR *in vitro* and in *vivo*; 4) The clinical significance of exosomal DANCR in osteosarcoma patients. Results: Expression of exosomal DANCR in osteosarcoma patients was obviously higher than in healthy controls. Expression of DANCR in the supernatants of osteosarcoma cell lines (Saos2, MG63, and HOS) was significantly increased compared to FOB cells. Furthermore, exosomal DANCR was stably expressed in serum of osteosarcoma patients, which was derived from tumor cells. Elevated expression of exosomal DANCR was further confirmed in 46 osteosarcoma patients, compared with bone benign tumors and healthy controls. Exosomal DANCR was obviously associated with initial metastasis (P=0.045). Additionally, at the cutoff of 2.52, exosomal DANCR significantly distinguished osteosarcoma from bone benign tumors and healthy controls (area under curves, 0.849), with 87.0% sensitivity and 66.7% specificity, superior to alkaline phosphatase. Moreover, patients with high exosomal DANCR levels had shorter overall survival rates than those in the low group (36.4% vs. 80.0%, P=0.008). Conclusion: Measurement of lncRNA DANCR in exosomes was shown to be a noninvasive tumor biomarker for diagnosis and prognosis of osteosarcoma patients.

Keywords: Osteosarcoma, lncRNA, exosome, DANCR, diagnosis, prognosis

Introduction

Osteosarcoma is the most common primary bone tumor in young adults and children, accounting for approximately 60% of sarcoma patients [1, 2]. Tumor metastasis remains an adverse factor for clinical prognosis in both high- and low-grade osteosarcomas. Generally, diagnosis of primary or metastatic tumors mainly relies on imaging examinations, including X-ray, magnetic resonance imaging (MRI), computed tomography (CT), and positron emission tomography (PET)-CT. Accompanied by the substantial development of surgical procedures and multi-chemotherapeutic regimens [3, 4], overall survival of patients has gradually increased over recent decades. However, clinical outcomes of patients remain unsatisfactory due to local recurrence or metastasis [5]. Therefore, there is an urgent need to develop novel detective methods for early detection of recurrence or metastasis, leading to improved clinical outcomes. Alkaline phosphatase (ALP) is a common serum-based biomarker for osteosarcoma, but it sometimes produces false positives because of an increase in children and interference of organ damage. Therefore, the selection of highly specific, sensitive, and noninvasive markers is the most important challenge in improving the tumor burden for osteosarcoma management.

Exosomes, originating from the endosomal compartment, are small membranous vesicles which function as messengers of intercellular communication [6]. Exosomes secreted from cells can transfer the signal by binding to the receptors on recipient cells. Its secretion is an evolutionary cellular mechanism as a cellular messenger [7]. Exosomes contain various types of RNAs, including long noncoding RNAs (lncRNAs). Recent evidence has shown that circulating exosomes play an important role in long distance intercellular communication by

Table 1. Baseline characteristics for osteosarcoma patients and healthy controls

Factors	Osteosarcoma patient N=69	Healthy control N=71	*P* for value[b]
Sex			
Male	44 (63.77)	36 (50.70)	0.103
Female	25 (36.23)	35 (49.30)	
Age (yrs)			
<25	41 (59.42)	41 (57.75)	0.841
≥25	28 (40.58)	30 (42.25)	
Smoking			
Yes	22 (31.88)	28 (39.44)	0.351
No	47 (68.12)	43 (60.56)	
Drinking			
Yes	29 (42.03)	25 (35.21)	0.407
No	40 (57.98)	46 (64.79)	
ALP			
Normal	39 (56.52)	-	-
Abnormal	30 (43.48)	-	
Tumor size (cm)			
<8	12 (17.39)	-	-
≥8	57 (82.61)	-	
Tumor site			
Tibia/Femur	49 (71.01)	-	-
Elsewhere	20 (28.99)	-	
Tumor stage[a]			
I	14 (20.29)	-	-
II	42 (60.87)	-	
III	13 (18.84)	-	
Chemotherapy			
Yes	20 (28.99)	-	-
No	49 (71.01)	-	
Initial metastasis			
Yes	18 (26.09)	-	-
No	51 (73.91)	-	

[a]Tumor stage based on the Enneking surgical stage. [b]Two-side Chi-squared test.

carrying lncRNAs [8]. Furthermore, several exosomal lncRNAs have been associated with tumor progression, which may function as meaningful markers [9, 10].

Recently, differentiation antagonizing non-protein coding RNA (DANCR) (ID: NONHSAG03-7936.2), a long noncoding RNA molecule, has been shown to suppress epidermal progenitor differentiation [11]. lncRNA DANCR has also been associated with the differentiation of mesenchymal tissues, such as odontoblast-like differentiation and chondrogenic differentiation [12, 13]. Furthermore, DANCR expression was positively correlated with a poor prognosis for osteosarcoma patients, as well as tumor invasion and metastasis [14]. However, it has not been applied for clinical screening purposes due to difficulty in getting tissue from suspicious patients with osteosarcoma. In view of these reports, it was hypothesized that measurement of exosomal DANCR might open a noninvasive approach for diagnosis and prognosis of osteosarcoma patients.

The present study systematically explored the existence, stability, and source of exosomal DANCR in the serum. Furthermore, this study investigated the clinical significance of exosomal DANCR using two independent cohorts, including patients with osteosarcoma, healthy controls, and bone benign tumors.

Materials and methods

Patient and sample processing

This study was divided into a training cohort and subsequent validation cohort. For the training cohort, all samples were obtained from Yifu Hospital of Nanjing Medical University to investigate the feasibility of exosomal DANCR. In the validation cohort, exosomal DANCR was further confirmed in an independent cohort of 46 hospitalized patients. There were 22 age-matched patients with other benign tumors and 45 healthy controls in Yifu Hospital of Nanjing Medical University and The Second Affiliated Hospital of Nanjing Medical University, between January 2009 and March 2012. Clinical characteristics of the training cohort are summarized in **Table 1**. Serum samples at post-operation were also obtained at 7-14 days after surgical resection. All serum samples were kept at -80°C until RNA extraction. Each patient was termly followed up by clinical visits or telephone calls every three months during the first years and every six months for the next three years. Written informed consent was obtained from each patient. All experimental protocols were conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Nanjing Medical University.

Cell lines and culture

Human osteosarcoma cell lines (Saos2, MG63, and HOS) and human osteoblast cell lines (FOB) were obtained from the Type Culture Collection

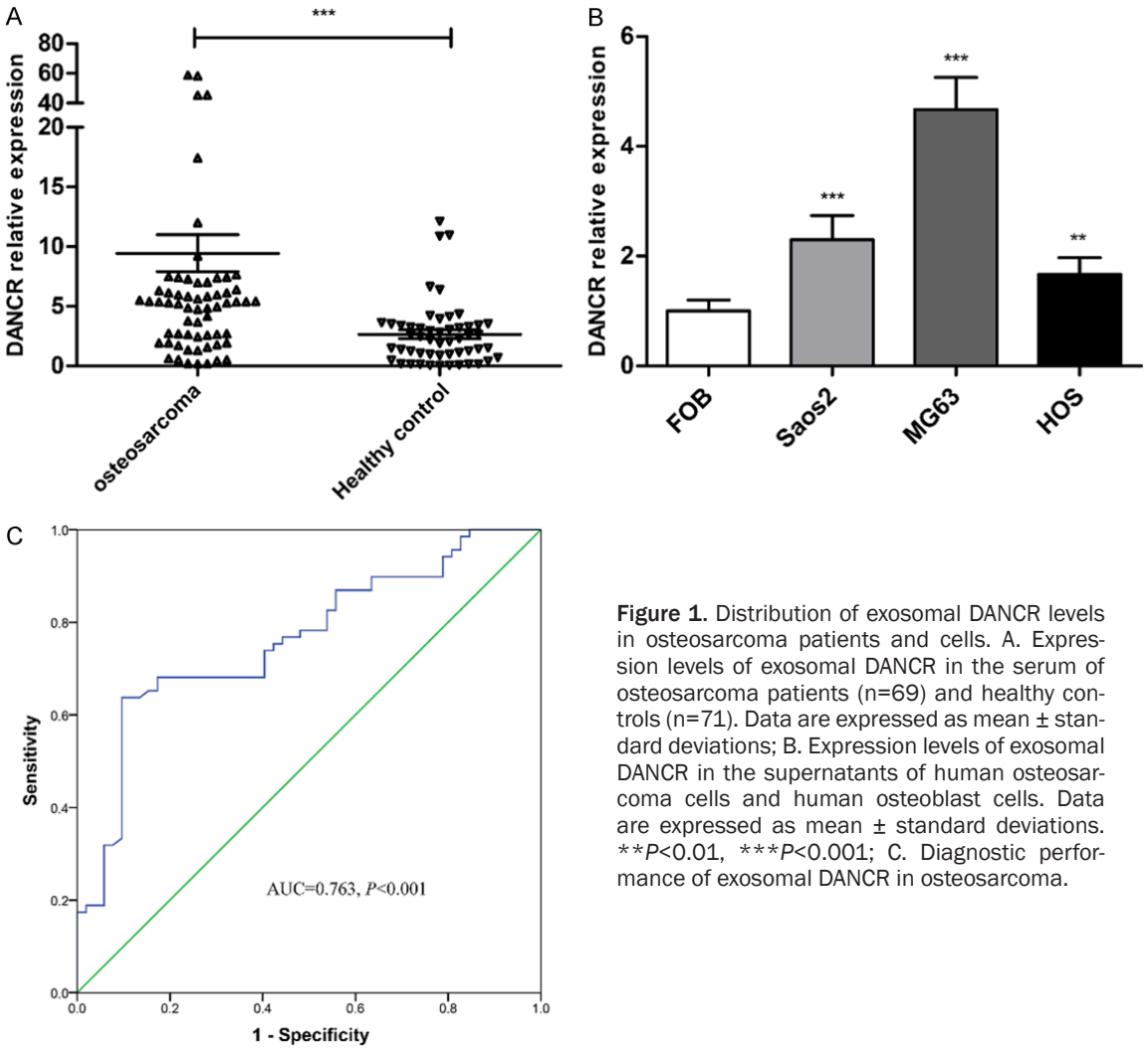

Figure 1. Distribution of exosomal DANCR levels in osteosarcoma patients and cells. A. Expression levels of exosomal DANCR in the serum of osteosarcoma patients (n=69) and healthy controls (n=71). Data are expressed as mean ± standard deviations; B. Expression levels of exosomal DANCR in the supernatants of human osteosarcoma cells and human osteoblast cells. Data are expressed as mean ± standard deviations. $^{**}P<0.01$, $^{***}P<0.001$; C. Diagnostic performance of exosomal DANCR in osteosarcoma.

of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium nutrient mixture (Hyclone, UT) added with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) in a humidified atmosphere with 5% CO_2 at 37°C.

Isolation of exosomal RNA

Blood samples from patients were centrifuged for 10 minutes at 1000 rpm to remove cell and debris. Supernatant was transferred and further centrifuged at 2000 rpm for 10 minutes to remove remaining debris. ExoQuicksolution (System Biosciences, Mountain View, CA) was added to the supernatant and then stored for 0.5 hours at room temperature. Exosomes were collected by centrifuging for 0.5 hours at 1500 g. Exosomal total RNA was extracted from exosome pellets by TRIzol Reagent (Thermo Fisher Scientific). Quality control of RNA was analyzed by NanoDrop spectrophotometer (Thermo Fisher Scientific).

Quantification of exosomal lncRNAs

Total RNA (100 ng) was synthesized to cDNA using the cDNA Reverse Transcription Kit (Takara, Dalian, China). Subsequently, quantification real time PCR (qRT-PCR) was performed by SYBR Green (Takara, Dalian, China) on an ABI 7500 Real-Time PCR Detection System (Applied Biosystems), according to manufacturer protocol. The ΔΔCt method was applied to calculate relative expression. Levels of lncRNA DANCR were normalized using GAPDH. Primer sequences were shown as follows: DANCR Forward: 5'-gccactatgtagcgggtttc-3'; Reverse: 5'-acctgcgctaagaactgagg-3'. GAPDH forward: AACGGATTTGGTCGTATTGGG; Reverse: CCTGGAAGATGGTGATGGGAT.

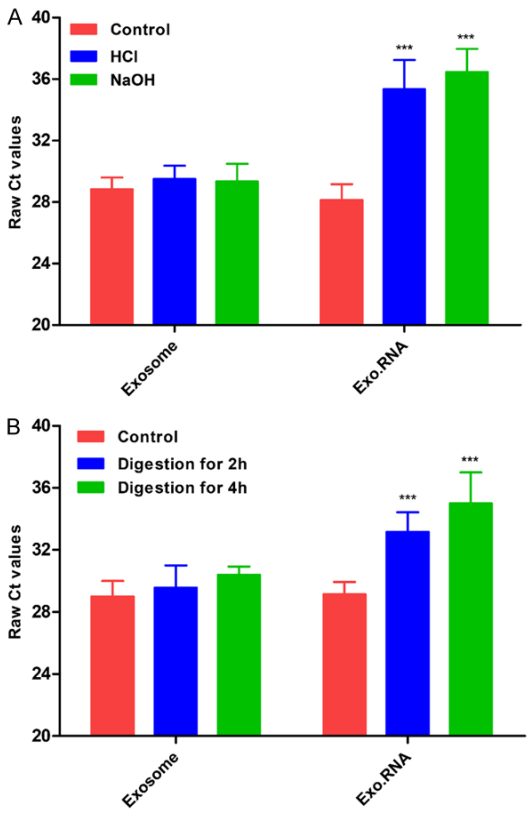

Figure 2. Stability of exosomal DANCR in serum. (A) Exosomal DANCR levels detected from serum exosome and exosome-depleted supernatant. Comparison of DANCR expression between exosome group and isolated nucleic acid (Exo.RNA) group when they were subjected to acid-base conditions and 2 or 4 h in RNase A (B). ***P<0.001.

Statistical analysis

One-way ANOVA or Student's t-test, along with Wilcoxon signed-rank test as appropriate, were applied to compare differences in serum lncRNA DANCR expression between osteosarcoma patients, age-matched benign tumors, and healthy controls. Optimal cutoff value for lncRNA DANCR was determined using X-tile software (Yale University, New Haven, CT, USA). X^2 test was used to evaluate differences between high- and low-level groups. Discrimination for osteosarcoma was determined by area under the curves (AUCs) and receiver-operating characteristic (ROC) curves. Kaplan-Meier curves and log-rank tests were applied to evaluate survival of patients. Significant prognostic indicators from log-rank tests were further included in multivariate Cox regression analysis. The nomogram associated with 5-year overall survival (OS) was depicted using R software via a stepwise algorithm and Harrell's concordance index (c-index) was applied to evaluate the performance of the nomogram. A calibration curve was generated to assess the nomogram's accuracy in predicting patient outcomes. P<0.05 is considered statistically significant.

Results

Baseline characteristics

Clinical features for patients with osteosarcoma and healthy controls are summarized in **Table 1**. A total of 44 (63.77%) men and 25 (36.23%) women were enrolled in the present study. Of these patients, 30 (43.48%) patients with increased levels of alkaline phosphatase (ALP) and 18 (26.09%) metastatic patients were recorded from newly diagnosed patients. According to Enneking staging criteria, the number of stages I, II and III was 14 (20.29%), 42 (60.87%), and 13 (18.84%), respectively. During the follow-up period, 49 (71.01%) patients stopped chemotherapy due to oppressive side effects, but 20 (28.99%) patients received system chemotherapy. Of these patients, 33 (47.83%) osteosarcoma died from cancer-specific disease. Median OS was 42 months.

Expression profile of exosomal DANCR levels in osteosarcoma patients and cells

To explore expression profiles of exosomal DANCR in osteosarcoma patients and cells, QRT-PCR was used to detect expression levels of exosomal DANCR from 69 osteosarcoma patients and 71 healthy controls. Results showed that DANCR relative expression in osteosarcoma patients was obviously higher than healthy controls (**Figure 1A**). Expression of DANCR in the supernatants of osteosarcoma cell lines (Saos2, MG63 and HOS) was significantly increased, compared to FOB cells (**Figure 1B**). Furthermore, exosomal DANCR was considered as a good biomarker to discriminate osteosarcoma patients from healthy controls, according to ROC curve analysis (**Figure 1C**).

Stability of exosomal DANCR

To explore the stability of exosomal DANCR in blood samples, exosome and exosomal isolat-

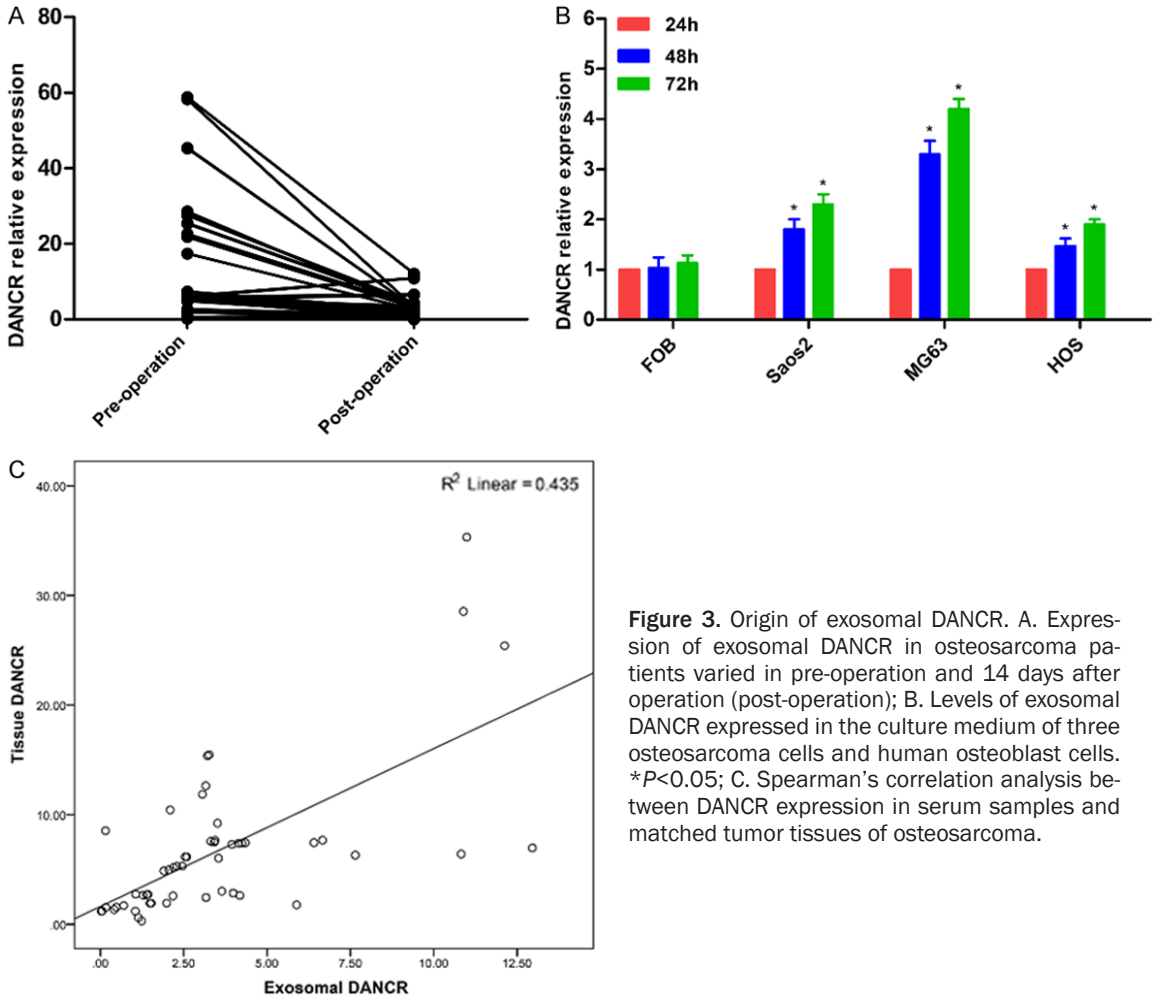

Figure 3. Origin of exosomal DANCR. A. Expression of exosomal DANCR in osteosarcoma patients varied in pre-operation and 14 days after operation (post-operation); B. Levels of exosomal DANCR expressed in the culture medium of three osteosarcoma cells and human osteoblast cells. **P*<0.05; C. Spearman's correlation analysis between DANCR expression in serum samples and matched tumor tissues of osteosarcoma.

ed DANCR were placed under harsh conditions, including acid-base treatment and incubation of RNase A. All samples were incubated with a strong acid or base for 2 hours (**Figure 2A**) and RNase A for 2 or 4 hours at room temperature (**Figure 2B**). Results showed that expression of exosomal DANCR in exosomes was not significantly changed under acid-base and RNase treatment, but the isolated DANCR from exosomes was completely degraded. Therefore, present data revealed that exosomal DANCR are stable and detectable in exosomes, providing an approach for osteosarcoma diagnosis as a feasible biomarker.

Origin of exosomal DANCR in serum

To determine whether serum exosomal DANCR was primarily released from the osteosarcoma cells, three independent experiments were performed. First, this study compared the expression of exosomal DANCR in paired pre-operative and post-operative serum samples. Data showed that expression of exosomal DANCR was significantly decreased in post-operative patients, compared with pre-operative patients (**Figure 3A**). Second, expression levels of exosomal DANCR were measured in three osteosarcoma cell lines (Saos2, MG63 and HOS) and one normal cell line (FOB), which were incubated for 1, 2 and 3 days. Data showed that exosomal DANCR could steadily increase over time in three osteosarcoma cell lines, but its expression hardly changed in FOB cells (**Figure 3B**). Third, DANCR expression was measured in exosome and corresponding osteosarcoma tissues to determine association of DANCR expression between the two groups (**Figure 3C**). Significant correlation was found between DANCR amplification in serum exosomes and matched osteosarcoma tissue samples (r^2= 0.435, *P*<0.001).

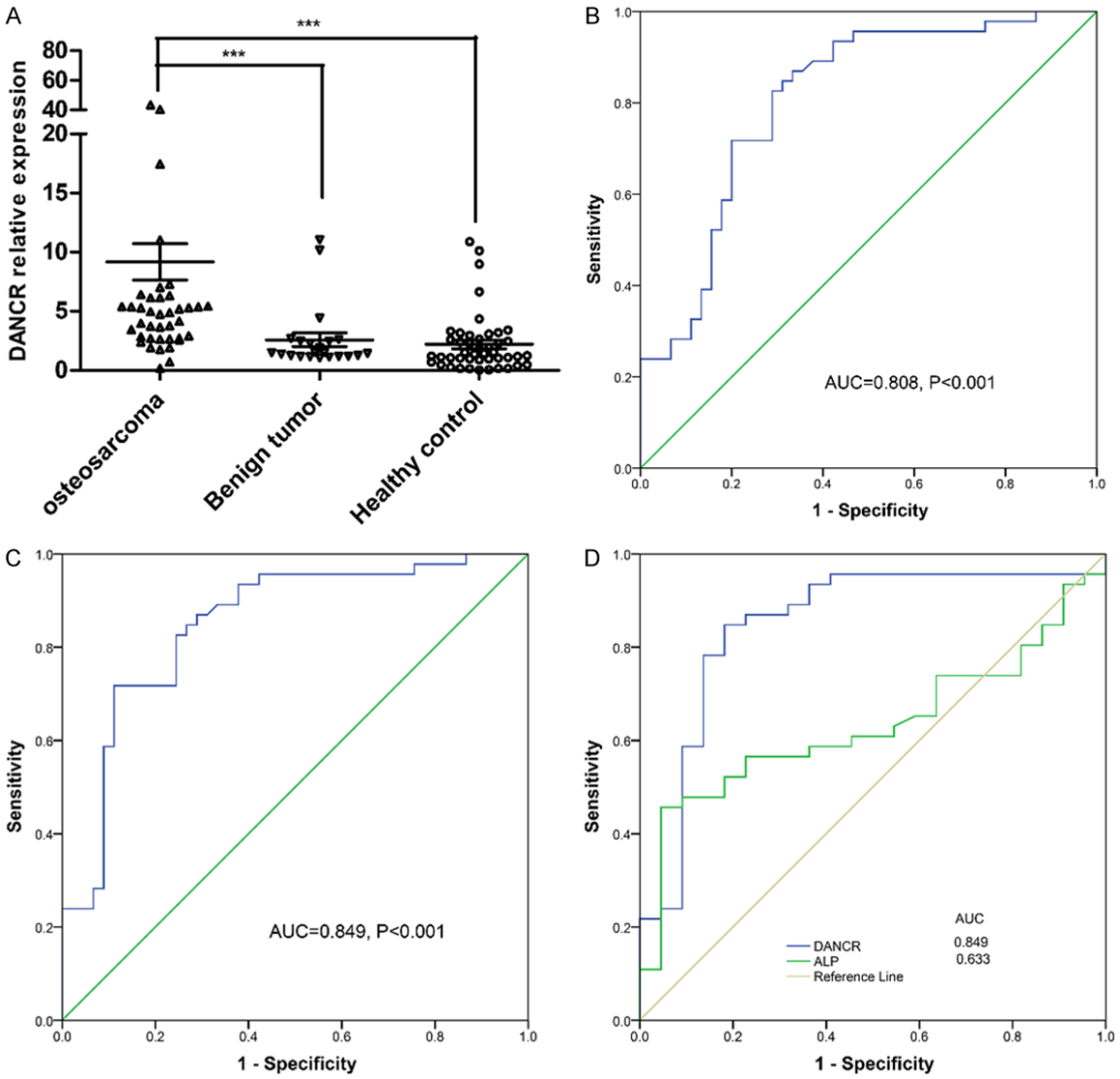

Figure 4. Expression of exosomal DANCR in validation phase. A. Relative expression of exosomal DANCR in osteosarcoma (n=46), bone benign tumors (n=22), and healthy controls (n=45); B. ROC curve for detection of osteosarcoma and healthy control using exosomal DANCR; C. ROC curve for detection of osteosarcoma and bone benign tumor using exosomal DANCR; D. Comparison of ROC curves for detection of osteosarcoma and bone benign tumor using exosomal DANCR and ALP.

Expression profile of exosomal DANCR levels in the validation phase

To further confirm expression profiles of exosomal DANCR in osteosarcoma patients, expression levels of serum exosomal DANCR were measured in an independent cohort of samples by RT-qPCR. Results showed a remarkable difference in exosomal DANCR expression among patients with healthy controls, other benign tumors, and osteosarcoma. Expression of exosomal DANCR was obviously increased in osteosarcoma, compared with healthy controls and other benign tumors (P<0.001, **Figure 4A**). However, no statistical differences were found between healthy controls and other benign tumor groups.

Diagnostic performance of exosomal DANCR for osteosarcoma

Exosomal DANCR has a strong ability for discriminating osteosarcoma from healthy control and benign tumor groups, with an AUC value of 0.808 and 0.849, according to ROC curve analyses, respectively (**Figure 4B** and **4C**). The sensitivity and specificity were 87.0% and 66.7% with an optimal cutoff value of 2.52.

Table 2. Association of DANCR expression with clinical characteristics in osteosarcoma patients

Factors	DANCR expression		*P* for value[b]
	Low expression N=25	High expression N=44	
Sex			
Male	15 (60.00)	29 (65.91)	0.624
Female	10 (40.00)	15 (34.09)	
Age (yrs)			
<25	16 (64.00)	25 (56.82)	0.559
≥25	9 (36.00)	19 (43.18)	
Smoking			
Yes	9 (36.00)	13 (29.55)	
No	16 (64.00)	31 (70.45)	0.580
Drinking			
Yes	7 (28.00)	22 (50.00)	0.075
No	18 (72.00)	22 (50.00)	
ALP			
Normal	18 (72.00)	21 (47.73)	0.051
Abnormal	7 (28.00)	23 (52.27)	
Tumor size (cm)			
<8	5 (20.00)	7 (15.91)	0.667
≥8	20 (80.00)	37 (84.09)	
Tumor site			
Tibia/Femur	21 (84.00)	28 (63.64)	0.073
Elsewhere	4 (16.00)	16 (36.36)	
Tumor stage[a]			
I	7 (28.00)	7 (15.91)	0.480
II	14 (56.00)	28 (63.64)	
III	4 (16.00)	9 (20.45)	
Chemotherapy			
Yes	6 (24.00)	14 (31.82)	0.491
No	19 (76.00)	30 (68.18)	
Initial metastasis			
Yes	3 (12.00)	15 (34.09)	0.045
No	22 (88.00)	29 (65.91)	

[a]Tumor stage based on the Enneking surgical stage. [b]Two-side Chi-squared test. High and low expression groups according to the optimal cutoff (2.52) of DANCR by ROC curves analysis.

Subsequently, this study compared diagnostic power with exosomal DANCR and conventional tumor biomarker ALP. The sensitivity and specificity were 45.7% and 67.8%, significantly lower than that for exosomal DANCR ($P<0.01$, **Figure 4D**), revealing that exosomal DANCR was superior to ALP in discriminating osteosarcoma from bone benign tumors. To further improve the diagnostic power for osteosarcoma, this study combined ALP with exosomal DANCR using the logistic regression model. ROC curve showed that the AUC for combination was 0.862, obviously increased compared with ALP or exosomal DANCR alone.

Association of exosomal DANCR with clinical characteristics and outcomes in patients with osteosarcoma

Association between exosomal DANCR and clinical characteristics of osteosarcoma patients is summarized in **Table 2**. All patients were divided into high and low expression groups, according to the cutoff value from ROC curve analysis. Results showed that high expression of exosomal DANCR was markedly associated with tumor metastasis. However, no significant association was observed between exosomal DANCR expression and other factors.

A total of 69 osteosarcoma patients were followed up, with a median OS of 42 months and 5-year OS rate of 52.2%. Kaplan-Meier curves showed that high expression of exosomal DANCR presented a significantly poor outcome, compared with low exosomal DANCR expression (**Figure 5**). This study further analyzed the clinical significance of clinical features via the Cox regression model. Statistical differences were observed between OS and exosomal DANCR, tumor stage, and initial metastasis, according to univariate analysis. Furthermore, significant factors in univariate analysis were included into the Cox regression multivariate analysis to determine whether exosomal DANCR was an independent predictor in osteosarcoma patients. Data showed that exosomal DANCR and initial metastasis maintained their significance as independent predictors for OS (**Table 3**).

Discussion

The present study is the first to explore the existence, origin, and stability of exosomal DANCR in the serum of osteosarcoma patients. Expression of exosomal DANCR was measured in patients with a variety of osteosarcoma presentations by RT-qPCR. Subsequently, this study compared its expression to those found in control groups with healthy controls and bone benign tumors. The present study observed remarkably increased levels of exosomal DANCR in the serum of osteosarcoma and,

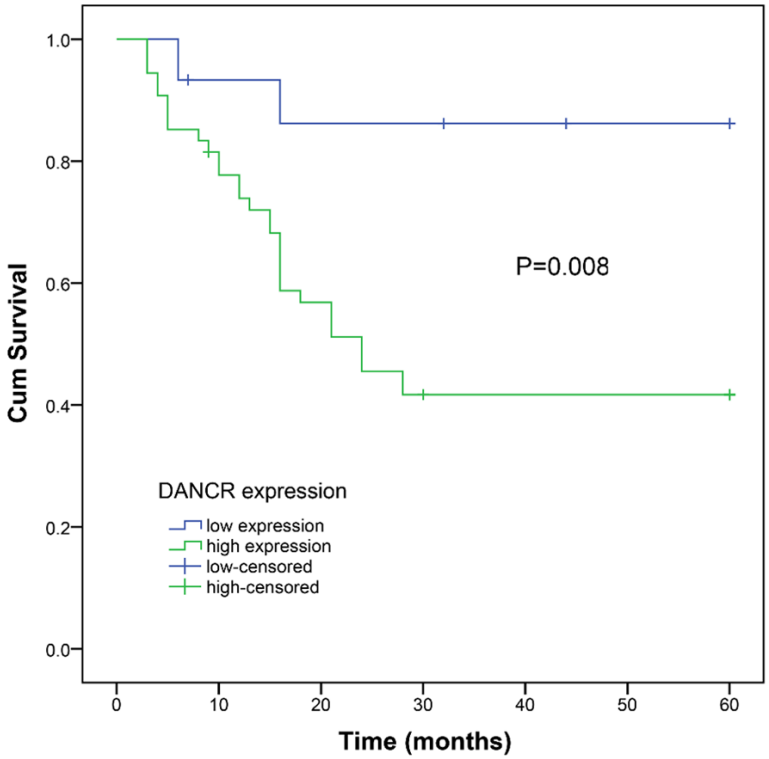

Figure 5. Kaplan-Meier curves for overall survival according to serum exosomal DANCR. The optimal cutoff (2.52) of exosomal DANCR was used to divide the osteosarcoma patients into high and low groups.

for the first time, confirmed its clinical significance in early diagnosis of osteosarcoma. Present data suggested that exosomal DANCR might be regarded as a good predictor for osteosarcoma.

Human blood has always been considered a non-invasive, pleasant, and convenient approach. Emerging evidence has suggested that circulating lncRNAs are good biomarkers for cancer diagnosis, such as MALAT1 [15], HOTAIR [16] and H19 [17]. The current study explored the stability of exosomal DANCR, suggesting that exosomal DANCR was significantly stable with treatment of acid-base and RNase A digestion. Therefore, it was confirmed that exosomes can prevent degradation of DANCR. Furthermore, it was observed that exosomal DANCR could be released into the cell culture medium at a measurable level over time. Subsequently, this study investigated the correlation of lncRNA DANCR expression between serum exosome samples and tissues matched for the same patients, detecting exosomal DANCR levels at pre-operation and post-operation. Data indicated that exosomal DANCR was released from tumor cells and could enter the peripheral blood. Thus, exosomal DANCR from blood, as a biomarker, is more feasible and stable than ever before.

Development of osteosarcoma is a multistep process, with dysregulation of genetics and epigenetic leading to tumor carcinogenesis. Early studies indicated that lncRNA DANCR could evaluate stemness features of hepatocellular carcinoma by inhibition of CTNNB1 [18]. Jiang et al. [14] suggested that lncRNA DANCR promoted osteosarcoma cell proliferation, migration, and invasion *in vitro* and *in vivo*. Mechanistically, DANCR can increase cancer stem cell function by promoting AXL via competitively binding to miR-33a-5p-PI3K-Akt signaling pathways. Present data demonstrated that upregulation of exosomal DANCR presents poor prognosis in osteosarcoma. Taken together, these results suggest that lncRNA DANCR plays an important role in osteosarcoma formation and development.

Recently, Jiang et al. [14] measured DANCR expression in tissues of osteosarcoma patients by RT-qPCR, finding that its expression was increased in tissue. The present study purified the exosome and observed elevated exosomal DANCR in serum of osteosarcoma patients, compared to healthy controls and bone benign tumors, consistent with previous conclusions. The present study also showed that exosomal DANCR levels were remarkably correlated with poor outcomes. Collectively, these data demonstrate that increased expression of exosomal DANCR might be associated with advanced osteosarcoma.

This study is the first report of the diagnostic performance of exosomal DANCR for osteosarcoma detection. Results demonstrated that exosomal DANCR significantly distinguished

Table 3. Univariate and multivariate Cox proportional hazards regression analysis of overall survival in osteosarcoma patients

Factors	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*P*	HR (95% CI)	*P*
Sex	0.67 (0.32-1.41)	0.287		
Age	1.94 (0.98-3.85)	0.057		
Smoking	1.29 (0.62-2.66)	0.497		
Drinking	1.37 (0.69-2.72)	0.369		
ALP	1.08 (0.93-1.25)	0.338		
Tumor size	2.62 (0.80-8.59)	0.112		
Tumor site	0.94 (0.43-2.01)	0.864		
Tumor stage (III)	5.05 (1.38-18.39)	0.014	2.63 (0.65-10.57)	0.173
Chemotherapy	0.56 (0.27-1.15)	0.113		
Initial metastasis	2.66 (1.33-5.35)	0.006	2.30 (1.08-4.91)	0.031
DANCR expression (High)	5.43 (1.30-22.71)	0.021	4.97 (1.15-21.44)	0.032

HR, hazard ratio; CI, confidence interval. Tumor stage based on the Enneking surgical stage.

osteosarcoma patients from bone benign tumors and healthy controls, with a high AUC value of 0.849, with a sensitivity of 87.0% and specificity of 66.7% at optimal cutoff of 2.52, indicating that exosomal DANCR may have superior discriminating power compared with ALP, a traditional circulating tumor marker for osteosarcoma. No association between exosomal DANCR and ALP was observed in serum. This study further observed whether discriminating power was improved by combination of exosomal DANCR and ALP. Interestingly, the AUC was obviously increased through combination of these two markers, showing a better diagnostic performance than that for ALP or exosomal DANCR alone. Therefore, results suggest that measurement of combination of exosomal DANCR and ALP might be a feasible complement to current osteosarcoma diagnostic strategies.

The present study also evaluated the prognostic power of exosomal DANCR, using Kaplan-Meier survival analysis. Results showed that osteosarcoma patients with high expression of exosomal DANCR had significantly decreased OS rates, compared to those with low levels. Furthermore, Cox regression analyses showed that exosomal DANCR was an independent predictor. Collectively, exosomal DANCR may serve as a precise biomarker for poor outcomes in osteosarcoma patients.

Conclusion

In summary, this is the first study to prove that exosomal DANCR complies with important characteristics of tumor markers. It is non-invasive and stable, with high sensitivity and specificity. Furthermore, exosomal DANCR is obviously associated with tumor progression and poor prognosis. Therefore, these findings may provide an avenue for development of an early diagnostic and prognostic biomarker for osteosarcoma.

Disclosure of conflict of interest

None.

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